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PURIFICATION OF N-ACETYLGALACTOSAMINIDASE BY ISOELECTRIC FOCUSI--ETC(U)
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PURIFICATION OF N-ACETYLGALACTOSAMINIDASE BY ISOELECTRIC FOCUSING.

by

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October 13, 1981

Dr. Arthur B. Callahan
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Dear Dr. Callahan:

This progress report covers the period from 1 November 1980 to 1 October, 1981 on contract N00014-78-C-0767 NR 207-143.

This project is aimed at the enzymatic conversion of type A (and B) red blood cells to type O (universal donor) cells. Activity during the period has been directed at purification of the enzymes from human placenta, assays for contaminating enzymes and development of quantitative assays for conversion of red blood cells. Azyme from humans is being studied because Azyme from non-human sources exposes red cells to potentially antigenic substances which might cause immune reactions in recipients.

I. Partially purified placental Azyme has no sialidase activity.

It is essential that any enzyme preparation used for converting type A cells to type O cells be free of sialidase (neuraminidase) activity since Aminoff et al (1977) have shown that sialidase treated erythrocytes are rapidly eliminated from the circulation and sequestered in the liver and spleen.

The sialidase activity of crude placental extracts and of the partially purified enzyme was determined using fetuin as substrate (Thomas et al, 1978). Sialic acid was determined by the procedure of Warren (1959). Assays were performed at pH 3.5, 4.5, 5.5 and 6.5.

Partially purified Clostridium perfringens Azyme was used as a positive control since it is known to contain sialidase. The clostridial enzyme was active at all of the pH's tested. Neither crude nor partially purified placental Azyme has sialidase activity at pH 6.5 or 5.5. This is encouraging since pH's alkaline to 6.5 will be used for red cell conversion. Crude homogenate had slight activity at pH 4.5 and 3.5. At pH 4.5 it had an activity of .0046 μ moles of sialic acid released per mg protein per hr. At pH 3.5 the activity was .0036. For reference, purified sialidase has an activity of 30 so that even crude extracts contain very little sialidase.

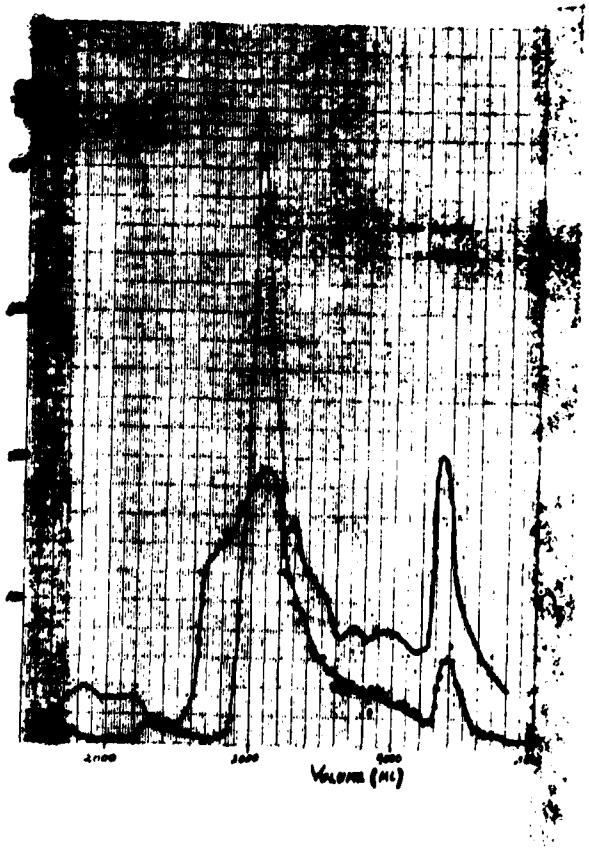
The partially purified enzyme obtained after ammonia sulfate fractionation and precipitation of impurities at low ionic strength was devoid of activity within experimental error yielding less than 3×10^{-4} μ moles of product/mg hr at pH 4.5 and less than 9×10^{-4} μ moles at pH 3.5. Dr. Aminoff also examined a placental Azyme preparation and found it free of sialidase.

The pH profile resembles that of lysosomal sialidase. This enzyme is fairly unstable (Aronson and de Duve, 1968) so that destruction of the enzyme during fractionation and storage may contribute to the absence of activity.

II. Further purification of placental Azyme.

After a number of small scale runs, a large scale purification of Azyme on DEAE cellulose was developed using a 5 x 90 cm column loaded with 20 to 25 g of partially purified enzyme which had been prepared by ammonium sulfate fractionation followed by precipitation of impurities at low ionic strength. The Azyme was eluted with a linear gradient of sodium chloride going from 0 to 0.5m at pH 6.5 in 0.01m phosphate buffer. A total of 6.6 liters was used for elution. In addition to the major peak, 3 minor peaks were found (Fig. 1).

Fig. 1 Chromatography of placental Azyme on DEAE. The volume of eluate is plotted against the concentration of protein (—) and Azyme (o—o)



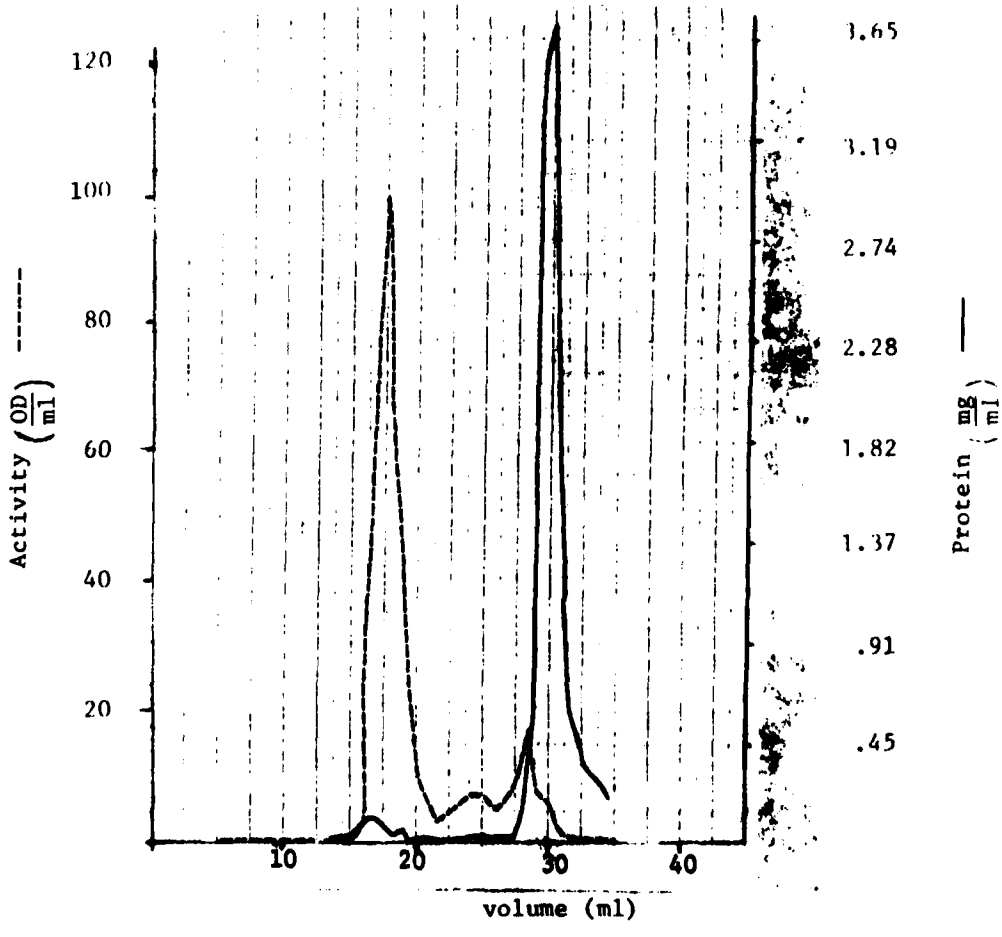
All are being concentrated so that they may be further purified and studied for their effect on type A and B red cells. So far the Azyme from about 100 placentas has been brought through this step. Overall recovery of enzyme activity was 59.6%. The yield and relative specific activity of the fractions are given in Table I.

Table I. Azyme Fractions Obtained by DEAE Chromatography

Peak	Relative Specific Activity	% of Input
A	2.3	0.4
B	6.3	1.6
C	36.4	29.4
D	7.4	4.9

Several small scale runs have been done with hydroxal apatite. In a typical run, a 1.0 x 14 cm column was loaded with 5.6 mg of protein from a euglobulin supernatant and eluted with a 40 ml gradient of sodium phosphate buffer going linearly from 1 mM to 50 mM (Fig. 2).

Fig. 2 Purification of Azyme on hydroxyapatite enzyme activity (----) and protein (——) are plotted against elution volume. Enzyme is purified 4600 fold over starting placental homogenate.



The specific activity of the peak tube was 4600 times the placental homogenate. Two methods of preparing hydroxal apatite were used, the Bernardi (1971) modification of the Tiselius procedure and the Spencer (1978) method. The resolution and flow rate were differed for the various hydroxal apatite preparations. Data given are for preparations made by the modified Tiselius procedure. Since the flow rate has been quite slow, we are now examining material made by the Spencer method. Experiments were also done to determine if batchwise elution would be successful. One ml of hydroxyl apatite was mixed with 0.5 ml of euglobulin supernatant (16.4 mg) and eluted successively with 2 washes of 2 ml each of the following pH 6.5 phosphate buffers. 1 mM, 5.5 mM, 10 mM, 25.5 mM, 50 mM, 100.5 mM and 200 mM. Thirty-five percent of the Azyme eluted at 25.5 mM with a specific activity of 291 u/mg and another 22% was in the second wash (specific activity 181 u/mg). The 2 fractions eluted at 10 mM each contained 9.5% of the material. The first elution at 50 mM contained 16.5% and the second 8% of the activity. The batchwise procedure is more convenient than the chromatography but the purification is much lower. Current work is directed at preparing hydroxal apatite with an improved flow rate and at upscaling the column process.

III. Attempts to develop assays for the activity of Azyme against red cells.

A major thrust of this period has been to determine whether placental Azyme is active in converting type A and B red cells to type O. Dr. Aminoff has tested a preparation of our placental Azyme and found it active against A substance. Weissman and Hinrichsen (1969) and more recently Sung and Sweeley (1980) have found that similar enzymes obtained from pork or beef liver hydrolyse A substance.

The environment and structure of the A reactive material of purified A substance differ from that of the erythrocyte. Hakomori (1981) has reviewed recent studies of erythrocyte A substance. Type A determinants occur on both glycolipids and glycoproteins. Four forms occur A^a, A^b, A^c and A^d. All contain an α 1-3 linked N acetylgalactosamine but differ in the internal sugars between the ceramide and glycoside terminus. A^b has two additional sugars inserted and A^c is branched containing two N acetylgalactosamine termini. A polysaccharide similar to A^a has been found to be linked to band 3 glycophorin, by a linkage which is unstable to base. Base stable type A substance has been found to be linked to asparagine of bands 3 and 4.5. It has a longer arm between the protein and the terminal N-acetylgalactosamine. Accordingly, we have attempted to develop assays using type A erythrocytes. Antisera to type A cells (typing sera), lectin and substrate competition have been used to attempt to follow the activity of Azyme on erythrocytes. None of the procedures have the required accuracy and sensitivity.

Two types of assay were investigated using anti A typing serum. In the absorption assay the volume of red cells required to reduce the titer was determined. For a serum active at a 1:512 dilution 10 to 20 µl of 0.5% cells usually reduced the titer to 1:128. Absorption with type O cells did not reduce the titer. A number of experiments were done using various sera (3 lots), different Azyme preparations, varying incubation times and temperatures and pH's of 5.5, 6.5, 6.8 and 7.2 using various buffers. Although we were very careful the assays had rather poor reproduceability and the results were inconclusive. Difficulty in reading the agglutination near the end point was a major factor causing difficulty.

A binding method was studied next. For this the antibody fraction of the typing serum was partially purified by precipitation with ammonium sulfate at 40% of saturation and labeled with ^{125}I by the Bolton and Hunter method (1973). Only a small number of counts (2% of input) bound to the cells when they were reacted with labeled antibody for 1/2 hr at room temperature. There was little specificity. Another aliquot of the partially purified antibody was further purified by gel filtration of Sephadex G25 and subsequent chromatography on DEAE. It was labeled by the procedure of Banerjee and Ekins (1961). This serum also lacked specificity. In a typical experiment 250 µl of a 0.10 dilution of labeled antibody was added to 250 µl of a 50% cell suspension of type A cells. O cells were used in a control tube. After incubation for 30 min at room temperature the cells were centrifuged and washed twice with saline. The pelleted A cells had 16,900 cpm and the O cells had 18,000 cpm.

The typing serum has a relatively small amount of specific antibody in it. It has a titer of 512 i.e. 2^9. In other experiments with hybridoma antisera to other antigens we have obtained titers of 2^{23} i.e. 8,400,000. The typing serum is pooled from hyperimmunized human donors and contains antibodies to all the minor blood group antigens (Rh, Kell, Duffy, Lewis, Kidd, Lutheran and many others). There appears to be sufficient antibody to these various determinants to mask any specific anti A binding.

We believe the best way to proceed is to prepare monoclonal antibodies to A substance. This project is underway in collaboration with Dr. Myron Leon. We have immunized several strains of mice and have found one which gives a good immune response. This aspect of our work is described in more detail in the accompanying proposal.

Binding of lectins labeled with ^{125}I was also studied as an assay method. Dolichos biflorus (horse gram) lectin which is most widely used for distinguishing type A cells from type O was ^{125}I labeled by the Bolton-Hunter procedure and separated from unreacted reagent by gel filtration. In experiments where 1.1×10^4 cpm in 0.1% gelatin were added to ten fold dilutions of type A and type O red cells starting with 10^8 cells no differences were observed in binding. About 50% of the added counts were bound when 10^8 cells were used. Incubation periods from 1/2 hr to 24 hr did not alter the specificity nor did attempts to absorb non-specific activity with O cells. Similar results were obtained with red cells from several different donors. (About 9% more counts bound to A cells than O cells.) The procedure of Hayes and Goldstein (1981) was also used to label the lectin as it has proven useful for lectins which contain sulphydryl sensitive groups. No differential binding of the labeled lectin to A or O cells could be demonstrated with cells incubated either at 0° or at room temperature for 20 hr. Serial dilutions of A and O cells were also tested against Dolichos biflorus lectin but the specificity was not improved. Bandeiraea simplifolica lectins BSI & II, the lectins from Glycine max (soybean) agglutinin type VI and Phaseolus limensis (lima bean) lectin have also been labeled by the method of Hayes and Goldstein. Product protection of the active site of the Phaseolus lectin during labeling did not improve specificity. Bandeiraea lectin was most promising giving 75% more counts bound to A cells than O.

A series of substrate competition experiments were done. If erythrocyte A substance is a substrate for Azyme and if its concentration is sufficiently high and its Km small enough, it should compete with p-nitrophenyl-α-N-acetyl-galactosamine. No competition was found under any of the following conditions:

A cells and O cells at various substrate concentrations and pHs, A cell and O cell membranes at different pH, substrate concentration, and membrane concentration, Triton solubilized membranes, Clostridium perfringens Azyme and membranes. The lack of competition in the latter experiment suggests that the concentration of A substance is too low since the K_ms for p-nitrophenyl-α-N-acetylgalactosamine (3.3 mM, Freeman (1981)) and Lipidlike substrates (e.g. Globotriaose, 4.1 mM, Dean & Sweeley (1979)) are similar for human Azyme.

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Freeman, S.L.: Characterization and modification of N-acetylgalactosaminidase from human placenta. M.S. thesis, Wayne State University, 1980.

The following publication was listed in my previous report as in press.

Rapaport, R.N., Jackiw, A. and Brown, R.K.: pH gradient flattening in isoelectric focusing in long polyacrylamide gels. Electrophoresis 1, 122-126 (1980).

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Enzymes which convert type A and type B blood group substances to type O, are being purified and tested as reagents for preparing type O, universal donor, red blood cells. Azyme from human placenta has been purified 1100 to 1300 fold using ammonium sulfate fractionation, isoelectric precipitation of impurities, and DEAE and hydroxyapatite chromatography. A number of assay procedures for accurately quantitating erythrocyte type A activity have been studied. These

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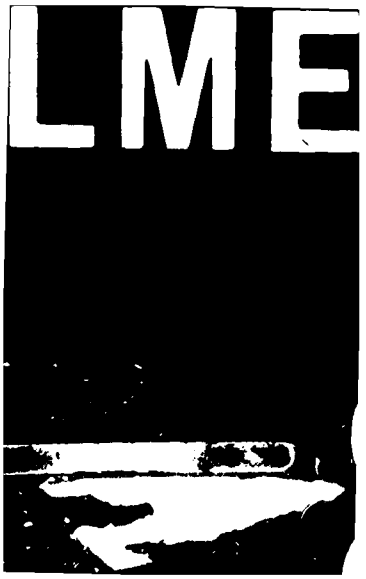
20. Abstract (continued)

include direct hemagglutin, absorption of antibody followed by hemagglutination with the residual antibody, substrate competition assays, and binding studies using a variety of radiolabeled lectins and antisera.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